Spleen Size Predicts Resistance of Rainbow Trout to *Flavobacterium psychrophilum* Challenge[1,2]

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Selective breeding of animals for increased innate resistance offers an attractive strategy to control disease in agriculture. However, this approach is limited by an incomplete knowledge of the heritability, duration, and mechanism(s) of resistance, as well as the impact of selection on the immune response to unrelated pathogens. Herein, as part of a rainbow trout broodstock improvement program, we evaluated factors involved in resistance against a bacterial disease agent, *Flavobacterium psychrophilum*. In 2005, 71 full-sibling crosses, weighing an average of 2.4 g, were screened, and resistant and susceptible crosses were identified. Naive cohorts were evaluated at 10 and 800 g in size, and most maintained their original relative resistant or susceptible phenotypes, indicating that these traits were stable as size increased >300-fold. During the course of these studies, we observed that the normalized spleen weights of the resistant fish crosses were greater than those of the susceptible fish crosses. To test for direct association, we determined the spleen-somatic index of 103 fish crosses; created high, medium, and low spleen-index groups; and determined survival following challenge with *F. psychrophilum* or *Yersinia ruckeri*. Consistent with our previous observations, trout with larger spleen indices were significantly more resistant to *F. psychrophilum* challenge; however, this result was pathogen-specific, as there was no correlation of spleen size with survival following *Y. ruckeri* challenge. To our knowledge, this is the first report of a positive association between spleen size and disease resistance in a teleost fish. Further evaluation of spleen index as an indirect measure of disease resistance is warranted. *The Journal of Immunology,* **2008, 180: 4156–4165.**

Infectious disease is a substantial source of loss in aquaculture (1, 2), and improved methods are needed to diminish this problem. Vaccines and antimicrobial agents are effective control methods for some pathogens. However, there are no commercial vaccines against many aquatic pathogens (3, 4), and antimicrobial use is limited by clinical drug resistance and by concern for the potential transfer of mobile resistance elements from agriculture-associated microorganisms to human pathogens (5). An additional approach to reduced disease loss is to selectively breed animals for increased disease resistance (6–8). Selective breeding is especially applicable to fish due to their high fecundity and relatively short breeding cycle. This allows the opportunity to examine large numbers of fish crosses for disease resistance using challenge tests while, at the same time, retaining a subset of unexposed cohorts for breeding (9). For this approach to be successful there needs to be significant heritable variation in broodstock disease resistance. In theory, once resistance is established, it would be cost-effective to maintain and thus advantageous for fish producers. A current limitation in implementation of selective breeding programs is the incomplete knowledge of the heritability, duration, and mechanism(s) of resistance, as well as the impact of selection on the immune response to unrelated pathogens.

One of the most important bacterial diseases affecting U.S. trout aquaculture is bacterial cold-water disease (BCWD).[5] The etiological agent of BCWD is *Flavobacterium psychrophilum*, which is also the cause of rainbow trout fry syndrome (RTFS) in small fish (10). Economic losses from *F. psychrophilum* are due to direct mortality and also to deformities in fish that survive infection (10, 11). There are several reasons why selective breeding for BCWD/RTFS is warranted: 1) the pathogen is widely distributed, and thus prevention is not feasible; 2) there is, at present, no commercial vaccine for BCWD or RTFS, and salmonids can be infected at the egg and fry stages (10, 12–14) before developing full immune system maturation and thus potentially compromising the utility of vaccination; 3) limited chemotherapeutics are availaible as only one drug, florfenicol is currently approved for treatment of BCWD in the U.S. (15); and 4) additive genetic variation for resistance to *F. psychrophilum* was demonstrated in a Danish rainbow trout population, indicating a favorable potential for selective breeding for increased resistance (16). In 2002, the National Center for Cool and Cold Water Aquaculture (NCCCWA) initiated a breeding program to increase rainbow trout growth rate and stress resistance (17). Because rainbow trout at our facility mature after 2 years of growth, two selection programs were initiated: selection for growth and stress resistance conducted in even-numbered years, and selection for disease resistance in odd-numbered years. In

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[5] Abbreviations used in this paper: BCWD, bacterial cold-water disease; RTFS, rainbow trout fry syndrome; SI, spleen somatic index; TYES, tryptone yeast extract with salts.

Table I. *Average weight (g) of rainbow trout (2005 year-class) used in this study*[a]

Trial	Challenge Dose (CFU/Fish)	Resistant Crosses				Susceptible Crosses			
		58	59	96	156	134	143	144	127
1	8.75×10^6	2.4[b]	2.6	2.5	2.6	2.6	2.7	2.5	2.2
2	1.24×10^7 (1.4-fold)	10.2 ± 2.0[c] (4.2-fold)	10.9 ± 1.4 (4.2-fold)	8.6 ± 2.3 (3.4-fold)	10.2 ± 2.0 (3.9-fold)	11.3 ± 2.6 (4.3-fold)	11.0 ± 2.8 (4.1-fold)	9.5 ± 2.2 (3.8-fold)	8.1 ± 2.5 (3.7-fold)
3	2.2×10^8 (25-fold)	789 ± 59[d] (329-fold)	720 ± 72 (277-fold)	801 ± 80 (320-fold)	ND[e]	842 ± 129 (324-fold)	890 ± 77 (329-fold)	850 ± 123 (340-fold)	ND

[a] Fold values represent the relative increase in dose of *F. psychrophilum* injected or weight of fish as compared to the first challenge.
[b] Average fish weight (g) derived from a pooled sample.
[c] Average fish weight (g) and S.D. (n=10 fish per cross).
[d] Average fish weight (g) and S.D. (n=12 fish per cross with the exception of cross 134 where only 10 fish were weighed).
[e] ND, not determined.

2005, we started the evaluation of survival following BCWD challenge, and initial phenotyping was conducted with fish from different crosses that averaged 2.4 g in size. In this population, disease resistance was also demonstrated to be a heritable trait that was not adversely correlated with growth performance (J. T. Silverstein, R. Vallejo, Y. Palti, C. E. I. Rexroad, T. Welch, G. D. Wiens, and V. Ducrocq, manuscript in preparation).

Herein, as part of a series of studies to understand disease resistance mechanisms in the NCCCWA broodstock population, we first examined whether the resistance or susceptibility phenotype was manifested throughout the growth cycle. We examined this by rearing unexposed siblings from crosses initially scored as resistant or susceptible and then challenging these fish after the average body weight had increased 4- and 300-fold. During the course of these studies, we observed that spleen weight of the naive, resistant fish was significantly greater than that of the naive, susceptible fish. In the second part of this study, we directly tested for association between spleen weight and resistance to i.p. challenge with

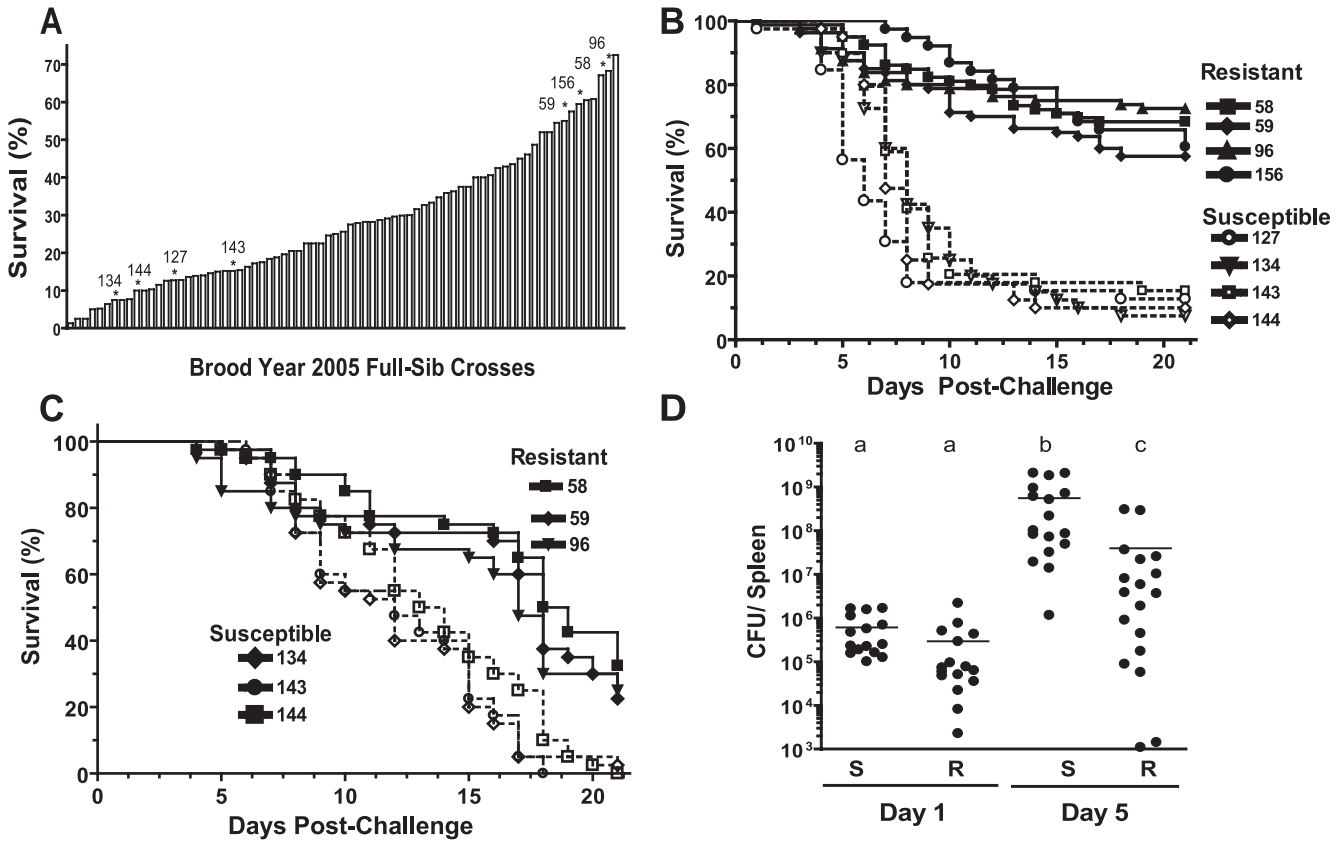

FIGURE 1. Survival of rainbow trout 2005 full-sibling crosses following i.p. challenge with *Flavobacterium psychrophilum*. *A*, Percentage survival on day 21 following challenge. Each column represents a separate fish cross ($n = 40$ or 80 fish per cross). *B*, Survival curves of selected crosses identified as either more "resistant" or "susceptible". These crosses are marked with an asterisk and by cross number in *A*. Fish averaged 2.4 g in size at the time of challenge. Susceptible crosses were significantly different ($p < 0.05$) from resistant crosses as determined by Kaplan-Meier survival analysis. *C*, Survival curves of resistant and susceptible crosses at 10 g in size ($n = 40$ fish per cross) following *F. psychrophilum* challenge. Data from replicate tanks per cross were pooled. Susceptible crosses were significantly different ($p < 0.05$) from resistant crosses as determined by Kaplan-Meier survival analysis, with the exception of cross 96 in relationship to cross 144 (adjusted $p = 0.06$; unadjusted for multiple comparisons $p = 0.002$). *D*, Total CFU per spleen determined on day 1 or 5 following challenge with 2.2×10^8 CFU *F. psychrophilum*. Fish size averaged 800 g. The susceptible group (S) contained either $n = 5$ or 6 fish from crosses 58, 59, and 96, and the resistant group (R) contain either $n = 5$ or 6 fish from crosses 134, 143, and 144. Significant differences are denoted by letters as determined by ANOVA with Bonferroni's multiple comparison post hoc test ($p < 0.05$).

Table II. *Pedigrees of eight trout crosses from the 2005 year-class selected for further study based on resistance or susceptibility to* F. psychrophilum *challenge*[a]

2005 Cross No.	Phenotype (at 2 g Weight)	2003 G_1 (Dam)	2003 G_1 (Sire)
58	Resistant	TL × TL	UW × UW
59	Resistant	TL × TL	UW × UW
96	Resistant	TL × HC	TL × S
156	Resistant	TL × TL	TL × UW
134	Susceptible	TL × TL	UW × UW
143	Susceptible	TL × UW	TL × HC
144	Susceptible	TL × UW	TL × TL
127	Susceptible	TL × S	S × HC

[a] The pedigree of the 2005 year-class fish can be traced back to the 2001 year class. Domesticated strains were transferred from various locations to the NCCCWA, and each founder fish has been designated by its source location. Year class 2005 fish are generation two (G_2), and the pedigree of their parents (G_1) and the founder population (grandparents, G_0) are listed. For the 2003 year-class, G_1 is listed in the two columns and the strain of the 2001 founder population (grandparents, G_0) is listed within a column. The strain of the dam is listed on the left and sire is to the right. For example, 2005 cross 96 maternal grandfather was HC. Abbreviations: UW, University of Washington strain; TL, Kamloops/Puget Sound steelhead; S, Shasta; HC, House Creek.

F. psychrophilum or *Yersinia ruckeri*. Potential resistance mechanisms and the application of these findings are discussed.

Materials and Methods

Animals

Rainbow trout crosses from the 2005 and 2006 year-classes were maintained at the NCCCWA and used at various growth stages (Table I). The original lines that contributed to the blend of the NCCCWA population are: 1) University of Washington, Donaldson (UW); 2) Kamloops/Puget Sound steelhead cross (TL); 3) College of Southern Idaho, House Creek (HC); 4) Ennis National Fish Hatchery Arlee strain (AR); 5) Ennis National Fish Hatchery Shasta strain (SH); 6) Kamloops strain originating from Kamloops region in British Columbia, Canada (KP); and 7) Idaho Department of Fish and Game, Hayspur strain (RP) (18). Fish were maintained in 12–14°C using well water with flow-through conditions and fed a standard commercial diet (Zeigler Bros). Tricaine methanesulfonate (MS 222, Sigma-Aldrich) was used to anesthetize the fish at 100 mg/L, and when necessary to euthanize the fish at 200 mg/L concentrations.

Bacterial strains

F. psychrophilum, strain CSF 259–93, was kindly provided by Dr. S. LaPatra (Clear Springs Foods). This strain is commonly used for challenge studies (19–21) and is representative of isolates causing disease loss in Idaho, a major location of rainbow trout production in the U.S. A clone of this strain was amplified on tryptone yeast extract with salts (TYES) agar (22), and a bank of −80°C frozen stocks was prepared from this culture to provide a standardized challenge source. DNA from this clone has also been subjected to genome sequencing (G. D. Wiens et al., unpublished data) and is part of a model system for studying host—pathogen–environmental interactions. For challenge, a single vial was thawed on ice and serial dilutions (PBS) were cultured on 150-mm TYES agar plates. Plates were incubated at 15°C and visually inspected after 5 days for culture purity. Cells were harvested from plates that contained ~75,000 CFU per plate (typically from the 1:100 dilution) and resuspended in chilled PBS (pH 7.2). Aggregates were dissipated by vortexing (30 s) four to five times during the course of 30 min. The OD at 525 nm was determined and adjusted to 0.65, which corresponded to $\sim 3 \times 10^8$ CFU/ml. For each experimental challenge, we determined the actual challenge dose by plating dilutions immediately before fish were challenged, and viable cell counts were determined after 5 days of incubation. The *Y. ruckeri* strain YRNC10*gfp* used in this study is a GFP-tagged derivative of a serotype 1 strain isolated from moribund rainbow trout collected at a fish farm in North Carolina (23). Bacteria were grown in brain heart infusion agar for 18 h to stationary phase, diluted 1/1000 in fresh media, and allowed to grow to a mid-log phase at 28°C before challenge.

Bacterial challenge

Animal procedures were performed under the guidelines of NCCCWA Animal Care and Use Committee Protocols 029 and 033. Challenge of fish from 71 full-sibling crosses is described (J. T. Silverstein et al., manuscript in preparation). For challenge at the 10 g size, fish were housed in 18-liter tanks. For challenge at the 800 g size, fish were housed in 100-liter tanks. Fish were challenged with an i.p. inoculum of *F. psychrophilum*, resuspended in PBS, and injected at the base of the pelvic fin. For 2005 year-class *F. psychrophilum* challenges, the challenge dose varied depending on fish weight (Table I). After growth to ~100 g, the water supply to cross 127 was inadvertently disrupted, and fish from this cross, as well as from cross 156, were not included in further studies. For the 2006 year-class challenge, four crosses of fish were pooled (10 fish per cross, a total of 40 fish per tank) and challenged i.p. with 2.1×10^7 CFU *F. psychrophilum* per fish. For *Y. ruckeri* studies, groups of 40 fish from the same crosses with high, medium, and low spleen indexes were pooled in 100-liter tanks and injected with 1×10^7 CFU of *Y. ruckeri*. Fish weighed an average of 99 g at the time of the *Y. ruckeri* challenge. A subset of moribund or dead fish was examined in each challenge trial using either Gram stain or plate culture to confirm the presence of the challenge pathogen.

Tissue harvesting and processing

In control fish, and also at various time points postchallenge, fish were euthanized and total body weight and spleen weight were typically measured within 30 min. When spleen sizes of 103 crosses from the 2006 year-class were measured, fish were euthanized and stored on ice for up to 4 h. Spleens were weighed using an AB104 balance (Mettler Toledo) to 0.001 g. Care was used to remove fat and connective tissue attached to the spleen. To determine the bacterial numbers in the spleen, a portion of each spleen was weighed and diluted 10-fold (w/v) in cold PBS. Tissue was disrupted by maceration with a 1-cc syringe before dilution and plating on TYES agar.

Spleen somatic index (SI)

Spleen indices were calculated by dividing spleen weight (mg) by total body weight (g).

Assessment of CFU from spleen samples

Dilutions of spleen cell suspensions were used to inoculate TYES-containing agar plates to culture *F. psychrophilum* or brain heart infusion agar plates to culture *Y. ruckeri*. For *F. psychrophilum*, plates were incubated at 15°C for 5–7 days before CFU were determined. For *Y. ruckeri*, plates were incubated at 28°C for 48 h before counting the CFU.

Statistical analyses

Survival curves were calculated using the product limit method of Kaplan and Meier using GraphPad v4.0 software. Survivorship curves for all crosses were compared using the log-rank tests (data from replicate tanks were pooled). For the 2005 year-class fish, a total of 28 pairwise comparisons were made in each of the *F. psychrophilum* challenges so that the p values were corrected for multiple comparisons using the formula $p_{\text{corrected}} = 1 - (1 - p)^n$, where n indicates the number of comparisons. For all other

Table III. *Survival analysis of eight selected crosses of fish challenged at an average size of 2.4 g*

	Resistant Crosses				Susceptible Crosses			
	58	59	96	156	134	143	144	127
Mortality/total	25/79	34/80	22/80	15/38	37/40	33/39	36/40	34/39
% Survival	68.3	57.5	72.5	60.5	7.5	15.4	10.0	12.8
Median survival (days)	ND[a]	ND	ND	ND	8	8	7	6

[a] Not determined, as 50% mortality was not reached by the end of the 21 day challenge.

Table IV. *Survival analysis of eight fish crosses challenged at an average size of 10 g*

	Resistant Crosses				Susceptible Crosses			
	58	59	96	156	134	143	144	127
Mortality/total	27/40	31/40	30/40	35/40	39/40	40/40	40/40	40/40
% Survival	32.5	21.9	25.0	12.5	2.5	0.0	0.0	0.0
Median survival (days)	18.5	18.0	17.0	15.5	12.0	12.0	13.5	17.0

comparisons, one-way ANOVA was used, and Bonferroni's multiple comparison was used as a post hoc test if the overall $p < 0.05$. Calculations were performed using GraphPad v4.0 software. CFU data were log-transformed before statistical analyses.

Results

Survival of rainbow trout following ip challenge with F. psychrophilum

The cumulative survival of 71 full-sibling crosses of rainbow trout was determined following *F. psychrophilum* challenge as part of a broodstock selection program (J. T. Silverstein et al., manuscript in preparation). The final percentage survival, at the end of a standard 21-day challenge period, ranged from 1.3 to 75% (Fig. 1*A*), and the SD between replicated tanks averaged 7%. Based on these results, naive cohorts from four crosses at each end of the survival distribution were selected for further analyses and denoted as either "resistant" or "susceptible" (Fig. 1*A*, crosses are marked by asterisks). These particular crosses were chosen based on four criteria: 1) they ranked among the top quartile of susceptible or resistant crosses (Fig. 1*A*); 2) they had similar average total body weights (Table I); 3) they shared, in some cases, common parents; and 4) sufficient numbers of uninfected cohorts remained for further study. Resistant crosses 58 and 59 shared a common dam (mother), as did susceptible crosses 143 and 144. The pedigrees of all trout crosses at the NCCCWA are tracked, and comparisons of the parentage of the four resistant vs the four susceptible crosses excluded the possibility that a particular founder strain contributed unequally to the resistant or susceptible phenotypes (Table II).

At an average of 2.4 g in size, the percentage survival of the four resistant crosses ranged from 57.5 to 72.5% and was higher than the susceptible crosses, which ranged from 7.5 to 15.4% (Fig. 1*B* and Table III). Each of the four resistant crosses were significantly different ($p < 0.05$) as determined by Kaplan-Meier survival analysis from each of the four susceptible crosses; within a category (resistant or susceptible), there were no significant differences between the four crosses.

To assess whether the resistance or susceptibility was manifested over the trout growth cycle, we challenged fish when they had increased in size either ~4-fold (10 g in size) or ~300-fold (800 g in size) (Table I). Mortality was the endpoint evaluation for the 10-g fish, and three of the resistant and three of the susceptible crosses of fish had the same relative ranking in survival (Fig. 1*C* and Table IV). For crosses 127 and 156, intermediate phenotypes

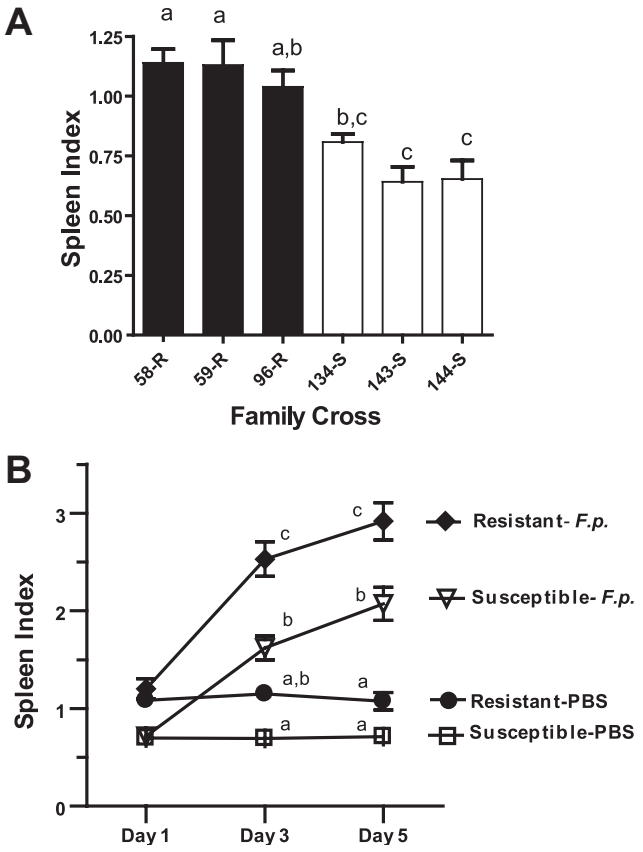

FIGURE 2. Spleen weight normalized to total body weight from resistant (58, 59, and 96) and susceptible (134, 143, and 144) trout crosses. *A*, Spleen index data by cross ($n = 15$ fish per cross, mean ± 1 SEM). Significant differences are denoted by letters as determined by ANOVA with Bonferroni's multiple comparison post hoc test ($p < 0.05$). *B*, Spleen indices of fish injected with either PBS or *F. psychrophilum* and sacrificed on days 1, 3, and 5 postchallenge. Values are mean spleen index ± 1 SEM ($n = 15$ fish per group with 5 fish from each cross).

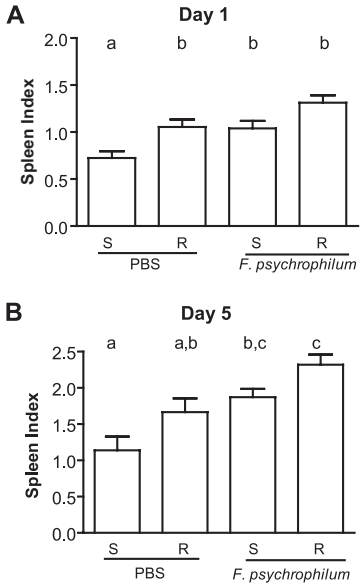

FIGURE 3. *A*, Spleen index values 1 day after injection with either PBS or *F. psychrophilum*. *B*, Spleen index values 5 days after injection with either PBS or *F. psychrophilum*. Significant differences are denoted by letters as determined by ANOVA with Bonferroni's multiple comparison post hoc test ($p < 0.05$).

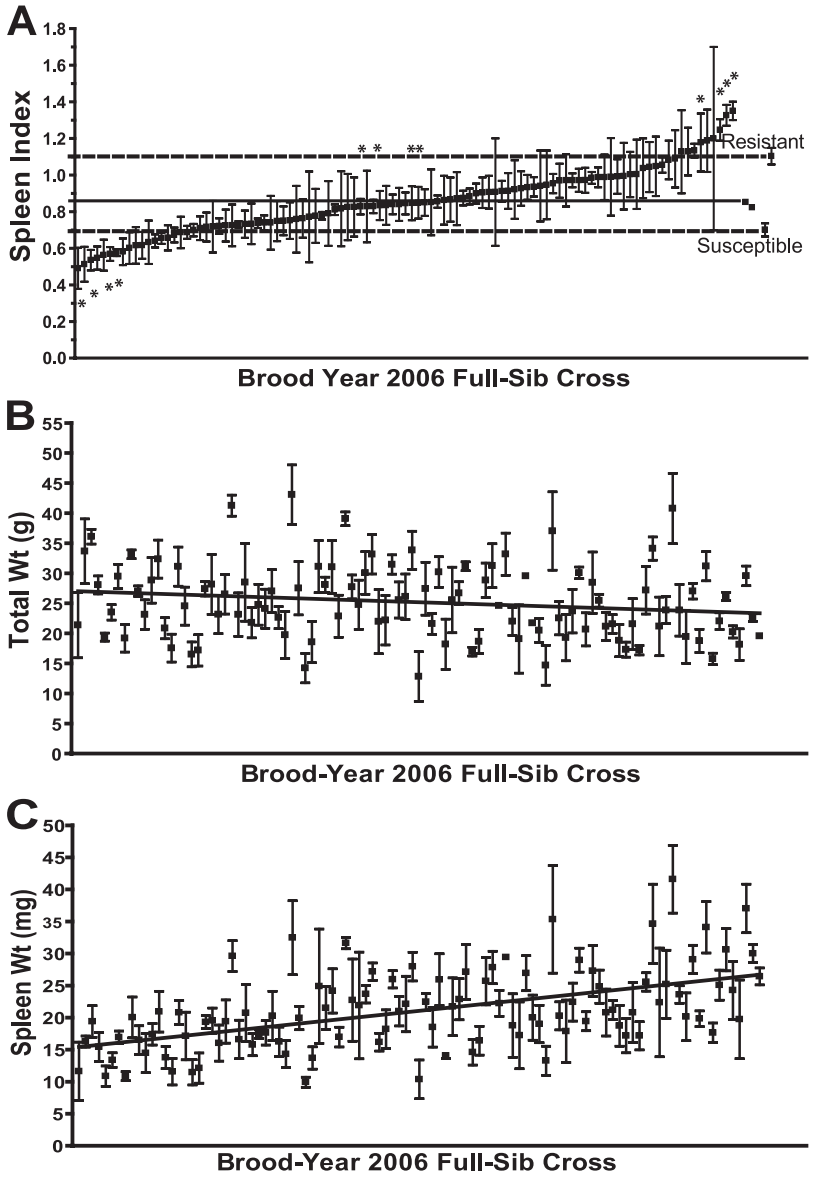

FIGURE 4. Evaluation of the spleen size of full-sibling crosses from the 2006 year-class. *A*, Spleen indices were determined for each cross ($n = 3$ fish per cross; mean ± 1 SEM are reported). Crosses are ordered from lowest to highest SI. The dotted lines indicate average SI ± 1 SEM of 45 susceptible or resistant fish determined from the 2005 year-class. Solid line is the average SI for the 2006 population ($n = 309$ fish). Asterisks denote crosses used in further studies. *B*, Average total body weight (±1 SEM) with crosses listed in the same order as in *A*. There was no correlation between body weight and SI ranking. *C*, Average spleen weight (±1 SEM) with crosses listed in the same order as in *A*. Spleen weight and relative ranking are positively correlated ($p < 0.0001$; $r^2 = 0.278$).

were observed, and because fish from cross 127 were inadvertently lost, these two crosses were not followed in further studies. The percentge survival of resistant crosses 58, 59, and 96 ranged from 21.9 to 32.5%, while the susceptible crosses 134, 143, and 144 ranged from 0 to 2.5% (Table IV). Each of the resistant crosses (58, 59, and 96) were significantly different as determined by Kaplan-Meier survival analysis from each of the susceptible crosses (134, 143, and 144), with the exception of crosses 96 and 144 ($p = 0.06$). When fish had reached an average of 800 g in size, fish were challenged and splenic bacterial load was determined on days 1 and 5 postchallenge. Splenic bacterial load was used as a surrogate measure of resistance, as mortality is not typically observed in adult fish following challenge. The data from each cross were analyzed separately, but because there were no significant differences between crosses within either the susceptible or resistant groups, the data were pooled due to the limited numbers of fish available from each cross at each time point ($n = 5$ to 6 fish per cross). On day 1 after challenge, there was a 2-fold higher average colony count in the susceptible fish that was not statistically significant ($p > 0.05$); however, by day 5, there was a 14-fold greater average colony count per spleen in the susceptible fish in comparison to the resistant fish ($p < 0.01$) (Fig. 1*D*). These data indicate that the fish identified as resistant at 2.4 g in size have a greater capacity to limit bacterial growth or trafficking to the spleen following challenge at 800 g in size.

Association of spleen index and resistance to F. psychrophilum *infection*

During the course of these challenge experiments, we observed a difference in the spleen size between naive-resistant and naive-susceptible animals. The mean spleen indices (spleen weight normalized to body weight) of resistant crosses were significantly higher than those of susceptible crosses of fish (Fig. 2*A*). As spleen enlargement (splenomegly) is known to occur following natural and experimental infection (24, 25), we next investigated if fish challenged with *F. psychrophilum* would still exhibit a difference in spleen size. As expected following challenge, infected fish (10 g size) displayed larger spleens than did unchallenged fish following infection (days 3 and 5, Fig. 2*B*). Interestingly, there remained a significant difference in spleen size between the challenged-susceptible and the challenged-resistant fish on days 3 and 5 postinfection. These results indicate that whether naive or challenged, the spleen index of fish from resistant crosses was larger than that of the treatment matched fish from susceptible crosses (Fig. 2*B*).

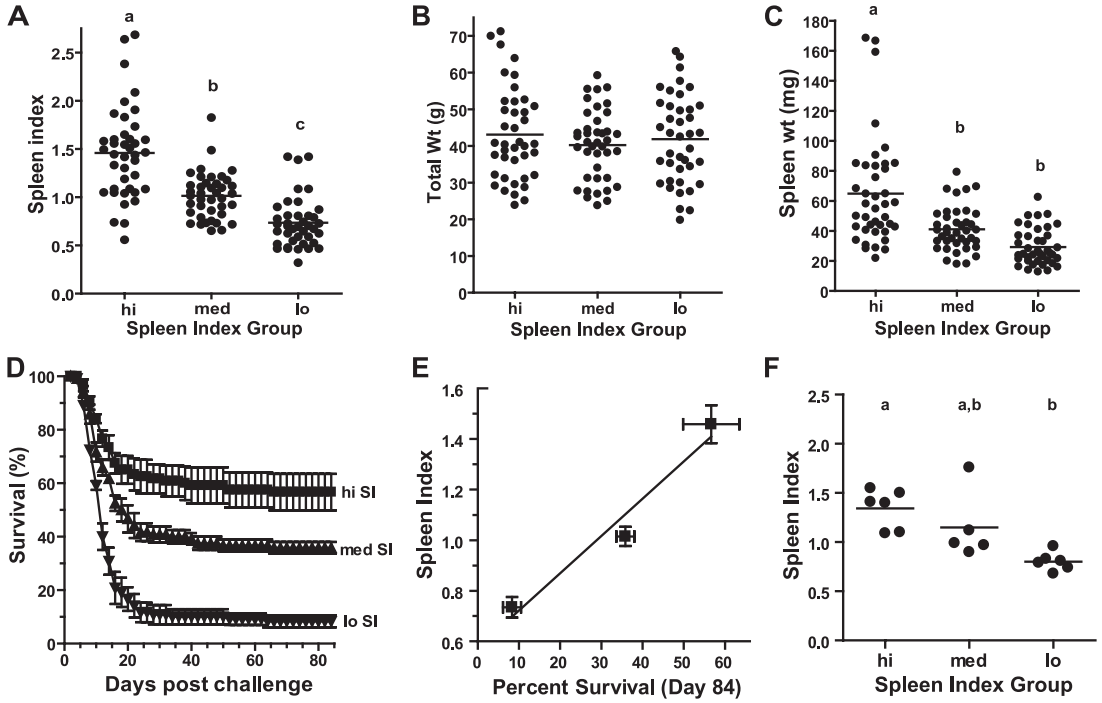

FIGURE 5. Spleen index predicts resistance to *F. psychrophilum* challenge. Twelve 2006 year-class crosses were segregated into three groups: high SI, medium SI, and low SI. *A*, Average spleen index of $n = 40$ fish (10 fish per cross). Each point represents an individual fish. Significant differences are denoted by letters as determined by ANOVA with Bonferroni's multiple comparison post hoc test ($p < 0.05$). *B*, Average weight of each group. *C*, Average spleen weight of each group. *D*, Percentage survival of high, medium, and low SI fish challenged i.p. with 2.1×10^7 CFU *F. psychrophilum* in 100 μl PBS. Results represent the average $\pm$ 1 SEM of triplicate tanks ($n = 40$ fish per tank). The three groups are significantly different ($p < 0.001$) from one another as determined by the Kaplan-Meier survival analysis. *E*, Linear correlation between day 84 survival ($\pm$1 SEM, n = triplicate tanks) and spleen index ($\pm$1 SEM, $n = 40$ fish per group). *F*, Spleen index of survivors 92 days postchallenge.

Similar trends, although not statistically significant, were observed between the 800-g naive and challenged fish on days 1 and 5 postchallenge (Fig. 3), indicating that the relatively larger spleen index of the resistant fish was maintained as fish grow, although increased variation in spleen size was also observed. Evaluation of postspawning female fish at the NCCCWA also showed a significant difference in spleen size between resistant (SI = 2.10 $\pm$ 1.08, mean and 1 SD, $n = 20$) and susceptible fish (SI = 1.18 $\pm$ 0.56, mean and 1 SD, $n = 10$). However, we also note that a cohort of susceptible and resistant fish held off-site that were predominantly males did not show a difference in spleen weight (data not shown), indicating that environmental differences or differences in sex may influence relative spleen size. In summary, these data support the notion that disease resistance and increased spleen size are consistent traits, albeit with some exceptions, exhibited over the lifespan of trout reared at our pathogen-free facility.

Evaluation of the spleen size of full-sibling crosses

At this point it was unclear whether susceptible fish had unusually small spleens, whether the resistant fish had unusually large spleens, or possibly a combination of both. Because we were unable to survey nonselected crosses from the 2005 year-class population, we reasoned that we would be able to indirectly address this question by sampling fish from the following year-class (2006). These fish were not directly related to the 2005 year-class, as trout have a 2-year breeding cycle; however, they should provide a relevant reference population to measure spleen size, as they were reared under similar conditions, had a similar founder strain background, and they were not part of the disease-resistance selection program. The spleen weight and total body weight of three fish from each of 103 full sibling crosses from the 2006 year-class were measured. The SI was calculated, and the crosses were ordered from lowest to highest SI (Fig. 4*A*). The population mean was 0.851 $\pm$ 0.25 ($\pm$1 SD, $n = 309$ fish) and, interestingly, the average SI of the 2005 susceptible fish 0.701 $\pm$ 0.242 (mean $\pm$1 SD, $n = 45$ fish) was less than the 2006 population average, while the resistant fish from 2005 had a higher SI of 1.103 $\pm$ 0.302 (mean $\pm$1 SD, $n = 45$; Fig. 4*A*, dotted lines). These data suggest that the susceptible fish have relatively small spleens and that resistant fish have relatively large spleens compared with the population average. There was no correlation ($r^2 = 0.030$) between body weight and SI ranking (Fig. 4*B*), but there was a significant positive correlation ($p < 0.0001$; $r^2 = 0.278$) between unadjusted spleen weight and relative ranking (Fig. 4*C*).

Evaluation of spleen weight, total weight, spleen index, and resistance to F. psychrophilum *challenge*

To directly test the relationship between resistance and spleen size, we hypothesized that if there is a direct link, we should be able to select fish based on spleen size and predict relative resistance to challenge. To test this hypothesis, three groups of fish were created based on their average spleen index value: high SI, medium SI, and low SI. Each group contained equal numbers of fish from four crosses. Before pooling, the SI of 10 fish from each cross ($n = 40$ per group) were measured to confirm the phenotype (Fig. 5). We examined the pedigrees of the high, medium, and low SI cross, and similar to the disease resistant/susceptible phenotypes, there was not a particular founder trout strain that was overrepresented in one of the SI groups (Table V). The three groups significantly differed in SI (Fig. 5*A*) but not in total weight (Fig. 5*B*). Unadjusted spleen sizes were also different between the high SI and the medium/low SI (Fig. 5*C*). Triplicate tanks for each group (each containing 10

Table V. *The pedigrees of 2006 year-class (G_3) small, medium, and large SI fish*[a]

2006 Cross No.	Spleen Index Group	2004 G_2 (Dam)	2004 G_2 (Sire)
4	Low	UW × UW:UW × UW	TL × TL:AR × AR
65	Low	TL × UW:TL × TL	UW × UW:TL × TL
84	Low	UW × UW:UW × UW	UW × TL:UW × UW
224	Low	TL × TL:TL × TL	UW × UW:UW × UW
26	Medium	TL × UW:TL × TL	UW × TL:TL × TL
82	Medium	UW × TL:TL × UW	TL × TL:UW × UW
139	Medium	TL × UW:TL × TL	TL × TL:UW × UW
207	Medium	TL × TL:TL × TL	UW × TL:UW × UW
138	High	TL × TL:UW × UW	TL × TL:TL × TL
161	High	TL × UW:TL × TL	UW × TL:UW × UW
225	High	TL × UW:TL × TL	UW × TL:TL × UW
230	High	RP:RP	TL × TL:UW × UW

[a] The pedigree of the 2006 class fish can be traced back to the 2000 year class. Domesticated strains were transferred from various locations to the NCCCWA and each founder fish has been designated by its source location. Year class 2006 fish are generation three (G_3) and their parents (G_2) are listed in the columns (G_2) with the origin of the dam in the left and the sire in the right column. The G_1 and G_0 fish origins are listed within each column. For example, for the 2006 brood year cross number 4, the origin of the sire (year class 2004 G_2) was a TL × TL (G_1) female offspring mated with an AR × AR male (G_1). Abbreviations: UW, University of Washington strain; TL, Kamloops/Puget Sound steelhead; AR, Arlee strain; RP, Hayspur strain.

fish from each of four crosses, $n = 40$/tank) were challenged with 2.1×10^7 CFU *F. psychrophilum*, and survival was monitored for 82 days (Fig. 5*D*). We used a longer challenge period in this experiment to ensure that complete mortality was reached. The survival ratios between groups were significantly different: high SI fish were the most resistant, followed by medium SI fish, and then low SI fish, which were the most susceptible (Fig. 5*D*). A positive correlation coefficient between spleen size and survival was observed that was surprisingly robust ($r^2 = 0.957$; Fig. 5*E*). To evaluate whether the surviving fish harbored detectable infection, five to six survivors from each of the three groups, at day 92 postchallenge, were assayed for spleen size and spleen CFU. No *F. psychrophilum* were cultured from any of these fish. Interestingly, in these survivors, the same relative difference in spleen size was observed, indicating that after an apparent clearance of an acute challenge, the spleen size returned to the original prechallenge mass (Fig. 5*F*). These data suggest that there is a difference in the homeostatic set-point governing spleen size between the high, medium, and low SI groups of fish. Note that as with the 2005 year-class, spleen size was a consistent family trait as fish grew in size. In 15 crosses of fish measured over a 50-day period during which fish weight increased 74% (from an average of 25.1 ± 6.2 to 43.7 ± 12.0 g), the average SI was significantly correlated between measurements (Pearson's r = 0.69; two-tailed p value = 0.0175).

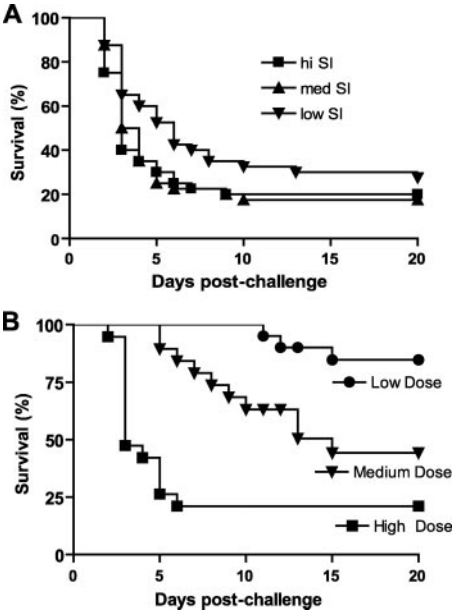

FIGURE 6. Equivalent survival of fish selected based on spleen size in response to i.p. challenge with *Y. ruckeri* strain NCDF. *A*, Survival of low, medium, and high SI fish following challenge. There were no significant differences between groups. *B*, Dose response of fish to *Y. ruckeri* challenge. Crosses were significantly different ($p < 0.01$) from one another as determined by Kaplan-Meier survival analysis.

Survival following IP challenge with Y. ruckeri

We next tested whether spleen size was correlated with resistance against an unrelated Gram-negative bacterial fish pathogen, *Y. ruckeri*. This is a strain that we have previously shown to cause splenomeglia following infection (26), and a small fraction of large granular cells in the spleen contain intracellular bacteria (23). High, medium, and low SI-grouped fish, from the same 2006 year-class families as the *F. psychrophilum* challenge, were injected with 10^7 CFU of *Y. ruckeri* per fish. Unlike the survival profile following *F. psychrophilum* infection, there was no significant difference between the three groups of fish following *Y. ruckeri* challenge (Fig. 6*A*). The failure to observe a difference was not due to the use of an overwhelming challenge dose, as dose-response experiments demonstrated that significantly different survival outcomes were obtained from 10-fold dilutions of *Y. ruckeri* (Fig. 6*B*). These data indicate that a large spleen size does not confer resistance to other Gram-negative pathogens, suggesting that resistance mechanism(s) may be pathogen specific in this cohort of fish.

Discussion

Herein, we characterize a BCWD resistance phenotype in rainbow trout and we begin to identify the factors that predict resistance. We demonstrate that most fish, within the crosses examined, maintained their relative resistant or susceptible phenotype as average body weight increased >300-fold. These results demonstrate that the resistance mechanism(s) are not transitory during fish development, and they suggest that our findings are relevant to a wide range of rainbow trout sizes. An interesting observation from these studies was that resistant fish crosses displayed larger-than-average spleen size, while susceptible fish had a smaller-than-average spleen size. When we selected fish crosses based solely on spleen

size, we found that this accurately predicted relative survival following pathogen challenge. We have further confirmed the link in a third year-class (year-class 2007) in a large challenge experiment using 3000 fish that was designed to identify disease resistance quantitative trait loci (Y. Palti, G. D. Wiens, unpublished results). In summary, our results support the presence of a heritable, genetic link between survival following *F. psychrophilum* challenge and spleen size in our rainbow trout population. This link is not a general resistance to Gram-negative bacterial infection, as there was no difference in survival following challenge with *Y. ruckeri*. Although a number of other studies have demonstrated heritable genetic variation in teleost survival in response to bacterial, viral, and parasitic challenge (6, 7, 9), to our knowledge this is the first report of a correlation between a physical trait (spleen weight) and bacterial disease resistance in a teleost fish.

How is spleen size linked to disease resistance?

The structure and function of the mammalian spleen has been well studied (27–29); however, much less is known about the cellular composition, development, and function of the fish spleen (30–33). Evolutionarily, the spleen is an organ exclusive to vertebrates, first appearing within the shark and bony fish lineages (28). In mammals, the spleen is the body's largest blood filter and it removes damaged or senescent cells unsuitable for continued circulation. It is also involved in the recycling and sequestration of iron, and it is crucial for the capture and destruction of pathogens and the formation of adaptive immunity (29). In rainbow trout, the spleen is a major source of Ab production (34, 35) and immunological memory (36); however, unlike sharks and some other teleosts, there is no evidence for a major role in erythropoiesis in the adult trout (37).

Although the precise mechanistic link between spleen size and disease resistance is unclear, we envision several possibilities. Fish with larger spleens may simply have a greater filtering capacity and thus increased immune function. In humans, the rate of clearance of ^{51}Cr-labeled heat-damaged red cells is positively correlated with the size of the spleen (38). It is well documented that the mammalian spleen is the crucial site required for the removal of encapsulated bacteria, as splenectomy increases the incidence of infections from encapsulated pneumococcus, *Hemophilus influenzae*, and meningococcus (39). Furthermore, in splenectomized mice and rats, clearance of injected bacteria is slowed compared with controls, resulting in fatal sepsis (40, 41). European starlings with larger spleens mounted stronger immune responses as measured by PHA responsiveness (42), and spleen size is a heritable trait in birds (42–45), although it is also influenced by environmental factors (46, 47). In our studies, we observed a surprisingly high correlation between average spleen index value and survival following *F. psychrophilum* challenge, suggesting a functional relationship (Fig. 5*E*). It is known that rainbow trout spleen size increases in proportion to total body weight (48, 49), and herein we have also observed that as fish increase in weight, an increased dose of pathogen is required to elicit mortality. Henryon and colleagues (16) also noted a correlation between trout body weight and disease resistance to *F. psychrophilum* challenge, with larger fish being more resistant. However, we cannot exclude the possibility that other developmental changes, such as a fully developed immune system, may contribute to increased resistance during growth. Keep in mind that there are examples where spleen size does not correlate with resistance; for example, C3H mice have larger spleens than do B6 mice (50), yet they are more susceptible to *Streptococcus pneumoniae* intranasal challenge (51).

Fish with larger or smaller spleens may reflect a specific overabundance or defect in a subset of cells that have critical immune function. This could involve specific recognition and/or killing of bacteria, cytokine production, or sequestering of an essential nutrient required for bacterial growth. In mammals, the spleen is considered to be uniquely specialized to filter bacteria as blood passes slowly through the dense meshwork of splenic cords, and thus the red pulp macrophages are able to remove poorly opsonized organisms much more efficiently than are other sites, such as the liver (52, 53). In the white pulp, marginal zone macrophages and marginal metallophilic macrophages are also crucial for bacterial clearance (54) and recognize microbes via specific ICAM-3-grabbing nonintegrin-related 1 (SIGN-R1) and a variety of other pattern recognition molecules (55, 56). It is possible that the larger spleens reflect a specific expansion of a macrophage-like cell type and thus are able to tip the balance of the infectious process. This hypothesis leaves unanswered why there was no difference following *Y. ruckeri* challenge, but it may relate to a different recognition or pathogenic process. It has previously been reported that resistance to *Y. ruckeri* and *F. psychrophilum* challenge are only weakly correlated genetically (16), suggesting that there are different mechanisms of resistance.

A larger spleen size may reflect a heightened baseline activation state or preexpansion of a responding cell population. The normal microbial flora is known to influence immune system development and activation state (57, 58). The immune responsiveness to pneumococcal infection in mice is influenced by the number and activation state of marginal zone B cells (59), and cytokines are critical for the expansion and maintenance of these cells (60). A number of cytokine genes have been identified that regulate cell populations within the spleen, and members of the TNF superfamily are particularly important in disease resistance (29, 51). We have recently reported the characterization of teleost TNF superfamily proteins and have identified seven genes that are expressed in the spleen and are thus of potential future interest (61). Many of the TNF superfamily proteins are involved in maintaining cellular homeostasis, and it is particularly noteworthy that both during an immune response and following clearance the relative size differences between the resistant and susceptible fish remained proportional, suggesting that the fish differ in a regulator governing a set-point of cell mass (Figs. 2*B* and 5*F*).

It is possible that spleen size may not be mechanistically responsible for the observed increase in resistance, but rather spleen size and resistance could both be controlled by tightly-linked, but functionally unrelated genes. We have begun to identify the genetic loci involved in *F. psychrophilum* challenge survival, and to date, four major histocompatability (MH) loci have been examined for association. The parents and grandparents of the 2005 year-class families were genotyped with markers linked to the four MH genomic regions (MH class Ia, MH class Ib, TAP-1, and MH class II) to assess linkage disequilibrium between those genomic regions and disease resistance. Markers from two of the four regions, MH class Ib and MH class II, were linked to challenge survival, although this analysis had limited statistical power due to a small sample size and thus should be considered suggestive for association. At present, the precise mechanistic link(s) between disease resistance, spleen size, and MH loci are unknown, but this is an area of interest that is being examined in a larger cohort of fish from the 2007 year-class. It is also noteworthy that the Hippo pathway, a highly conserved signaling pathway in *Drosophila* and mice, has recently been identified as a potent regulator of organ size (62), but a genetic link between this pathway and disease resistance has not yet been established to our knowledge.

Can spleen size be used as an indicator of disease resistance potential for selective breeding?

A major need in selective breeding is the development of easy-to-measure surrogates of disease resistance (6). This would reduce the

need for difficult and time-consuming challenge experiments and would reduce the problem of biosecurity at facilities focusing on rearing improved germplasm. The close correlation between spleen size and resistance suggests that spleen size measurements warrant further evaluation as an indicator for selective breeding; that is, such measurements are easy to determine and do not require sophisticated equipment. However, there are a number of potential factors that may influence spleen size that need to be addressed before we can recommend that such a strategy be adopted. The primary concern is that there are several environmental factors that influence spleen size in trout that may also affect size comparisons in unforeseen ways. Importantly, the fish used in these studies were reared under pathogen-free conditions under controlled temperature and photoperiod conditions, and they were handled and euthanized using consistent procedures. Even so, there was still considerable variation in spleen size between fish belonging to the same family cross. The salmonid spleen is rich in adrenergic innervations (63), and thus the stress response may impact measurements (64). In some mammals such as dogs and horses, the spleen serves a storage function to hold erythrocytes. In classic experiments with dogs, exercise induced contraction of the spleen to about one-half to one-third of its resting size (65). The rainbow trout spleen also has been reported to have a reservoir function, and it is likely that our sampling procedures induced reservoir erythrocyte release from the spleen (66). Relatively minor capture and handling stress results in rapid spleen size change (64, 66). Thus, our measurements likely reflect a minimum spleen size. Dietary components are also known to influence spleen size, as rainbow trout fed a vitamin E-deficient diet have enlarged spleens (67). Seasonal and temperature differences may impact spleen weight, as has been observed in brown trout (68). In mammals, it is well known that surgical or traumatic shock may cause the spleen to contract, while deep anesthesia may cause it to relax (69). Also, there is sexual dimorphism in spleen size in some birds and mammals (70, 71), but this has not, to our knowledge, been systematically examined in fish. Thus, it is important that if spleen measurements are to be used as a surrogate marker for resistance, pathogen exposure should be minimized, fish should be reared under common environmental conditions, and fish should be subjected to similar handling procedures. Until the molecular basis for the connection of spleen size to disease resistance is better defined, using spleen size as selection criteria should be approached with caution.

In conclusion, we have characterized the phenotype of *F. psychrophilum* resistant and susceptible families of fish as they increased in size >300-fold, and we have demonstrated a positive correlation between disease resistance and normalized spleen weight. We consider this to be an informative model system for defining the role of the teleost spleen in immunity to bacterial infection. More research is needed to define the molecular link between spleen size and resistance and to compare the heritability of spleen size in our population of fish with those of other domesticated populations of rainbow trout. Future studies will evaluate whether the selected fish are resistant to other strains of *F. psychrophilum* and routes of challenge, and ultimately whether they show improved performance in field trails.

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Disclosures

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